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ORIGINAL ARTICLE

From laboratory to the field: biological control of *Fusarium graminearum* on infected maize crop residues

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Abstract

Aim: To evaluate biological control agents (BCAs) against *Fusarium graminearum* on infected maize stalks as a means to reduce Fusarium head blight (FHB) in subsequently grown wheat.

Methods and Results: In the laboratory, BCAs were applied against *F. graminearum* on maize stalk pieces. *Clonostachys rosea* inhibited the perithecia development and ascospore discharge when applied before, simultaneously with and after the pathogen. In the field, we simulated a system with high disease pressure, that is, a maize-wheat rotation under no-tillage, by preparing maize stalks inoculated with *F. graminearum*. The infected stalks were treated with formulations of *C. rosea* selected *in vitro* or the commercial BCA strain *Trichoderma atrobrunneum* ITEM908 and exposed to field conditions over winter and spring between winter wheat rows. Monitoring with spore traps and of FHB symptoms, as well as quantification of *F. graminearum* incidence and DNA in harvested grain revealed significant reductions by *C. rosea* by up to 85, 91, 69 and 95% compared with an inoculated but untreated positive control, respectively. Deoxynivalenol (DON) and zearalenone (ZEN) contents were reduced by up to 93 and 98%, respectively. Treatments with *T. atrobrunneum* were inconsistent, with significant reductions of DON and ZEN under warm and wet climatic conditions only.

Conclusions: The findings support the application of *C. rosea* against *F. graminearum* on residues of maize to suppress the primary inoculum of FHB.

Significance and Impact of the Study: As sustainable agriculture requires solutions to control FHB, hence, the application of *C. rosea* during the mulching of maize crop residues should be evaluated in on-farm experiments.

Introduction

The ascomycete fungus *Fusarium graminearum* (teleomorph *Gibberella zeae*) is one of the world's most noxious plant pathogens and the main causal agent of the devastating cereal disease Fusarium head blight (FHB), affecting not only the yield of the crop, but more importantly, contaminating the harvested grain with dangerous mycotoxins (Dweba *et al.* 2017). The FHB complex also involves other toxin-producing *Fusarium* species such as *F. culmorum*, *F. avenaceum*, *F. cerealis*, *F. poae* and the nontoxigenic *Microdochium* species complex (Parry *et al.* 1995). The most prevalent mycotoxins in wheat are the type B trichothecenes deoxynivalenol (DON), its acetylated derivates 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol as well as nivalenol (NIV) which are produced by *F. graminearum* (Vogelgsang *et al.* 2019). Deoxynivalenol is an inhibitor of ribosomal protein synthesis, which results in high toxicity for humans and animals (Desjardins 2006). Besides DON and NIV, *F. graminearum* can also produce

the estrogenic mycotoxin zearalenone (ZEN), causing alterations in the reproductive system (Escrivá *et al.* 2015). Consequently, the European commission has set maximum limits to regulate the use of foodstuff contaminated with these mycotoxins. For unprocessed wheat and barley grains, these are 1·25 mg kg^{-1} DON and 0·1 mg kg^{-1} ZEN (European Commission 2006).

The infection with *F. graminearum* is highly correlated with the abundance and the type of host crop residues remaining on the field after harvest. On cereal crop residues, the pathogen survives saprophytically over winter, before it performs the next host passage in the following season (Dill-Macky and Jones 2000). In particular, the residues of maize, which are present in large amounts, provide an excellent substrate to develop and produce spores (Khonga and Sutton 1988; Blandino *et al.* 2010). Previous studies have shown the high disease potential of maize before wheat crop rotations when no-tillage practices were implemented (Edwards and Jennings 2018; Vogelgsang *et al.* 2019). On the maize substrate, the sexual ascospores are produced in perithecia and are then discharged into the air where they are dispersed with the wind to reach and infect the cereal head of their next host. The airborne ascospores represent the primary inoculum for the infection during flowering of wheat, when susceptibility of the crop is highest (Trail 2009).

Currently, measures against *F. graminearum* include the rotation with nonhost crops, conventional tillage to burry infected crop residues and the application of fungicides during flowering (Wegulo *et al.* 2015). However, due to the short time window for fungicide application, the efficacy of chemicals is highly variable and certain compounds, such as strobilurins, lead even to an increase of mycotoxins in the harvested grain (Forrer *et al.* 2000; Blandino *et al.* 2006; Paul *et al.* 2008). Clearly, the current methods come with substantial economic or environmental trade-offs and there is a need for alternatives to control FHB.

The control of *F. graminearum* with biological control agents (BCAs) that are capable to outcompete the pathogen on infected crop residues, has gained a lot of interest with the aim to reduce the primary inoculum in cereal-based crop rotations and thereby to minimize the use of chemical fungicides (Legrand *et al.* 2017). Mycoparasitic fungi belonging to the genera *Clonostachys* and *Trichoderma* are recognized among the best BCAs to control *F. graminearum*. The soil borne *C. rosea* has shown significant effects against *F. graminearum* by limiting the colonization by the pathogen on wheat stubbles (Luongo *et al.* 2005; Palazzini *et al.* 2013). Moreover, *C. rosea* inhibited the production of perithecia on leaf disks *in vitro* and significantly reduced the number of perithecia on infected maize kernels and wheat spikelets exposed to field conditions (Xue *et al.* 2009). Schöneberg *et al.* (2015) investigated *C. rosea* against *F. graminearum* in parallel to several other promising BCAs and concluded that only the application of *C. rosea* following an infection of wheat stalks with *F. graminearum*, significantly inhibited the development of perithecia. The developing perithecia showed a reduced size and flat appearance and did not discharge ascospores. Studies on the interaction between *C. rosea* and a wide range of fungal pathogens identified necrotrophic mycoparasitism, defined as the direct competition between fungi in which one is gaining nutrients from the dead cells of the other, as the primary mode of action. For *C. rosea*, this process involves the production of fungal cell wall-degrading enzymes and antibiotics to kill the host cells as well as an increased resistance against toxic secondary metabolites such as mycotoxins (Chatterton and Punja 2009; Rodríguez *et al.* 2011; Nygren *et al.* 2018). The secondary modes of action include the induction of plant defence responses and plant growth promotion in wheat (Roberti *et al.* 2008).

Similarly, the soil borne *Trichoderma atrobrunneum*, a member of the *Trichoderma harzianum* species complex (Chaverri *et al.* 2015), was identified as a potent competitor on infected crop residues against ascomycete pathogens, inhibiting for example the conidia production of *Stemphylium vesicarium*, causal agent of brown spot of pear, in the field (Rossi and Pattori 2009). Altomare *et al.* (2017) reported between 80 and 100% inhibition of perithecia development by *F. graminearum* on carrot agar when confronted with *T. atrobrunneum*. The molecular characterization of the commercial BCA strain *T. atrobrunneum* ITEM908 (formerly *T. harzianum* ITEM908) revealed several genes involved in mycoparasitic interaction and genes associated with an increased tolerance to toxic secondary metabolites produced by microbial competitors such as *F. graminearum* (Fanelli *et al.* 2018). Previously, mycoparasitic attack by *T. harzianum* against *F. graminearum* was investigated by scanning electron microscopy showing the inhibiting effect on perithecia formation and maturation on inoculated wheat straw (Inch and Gilbert 2007).

The objective of this study was to examine the potential of well-characterized strains of *C. rosea* and *T. atrobrunneum* to suppress *F. graminearum* on infected maize crop residues and thereby reduce the accumulation of DON and ZEN in wheat. To the best of our knowledge, no study has yet investigated the effects of crop residue treatments with BCAs on the aerial dispersal of *F. graminearum* in the field and the resulting accumulation of mycotoxins in the following crop. In a 'lab to field' approach, we first investigated the ability of *C. rosea in vitro* on maize stalk pieces infected with *F. graminearum* and compared the effects against a set of BCAs previously identified by Schöneberg *et al.* (2015).

Subsequently, we used a semi-artificial inoculation procedure that simulates the natural disease pressure of a maize-wheat crop rotation and applied formulations of *C. rosea* as well as the commercial BCA *T. atrobrunneum* onto infected maize stalks. The treated maize stalks were exposed to field conditions over winter and throughout spring between rows of two different winter wheat varieties. The airborne inoculum during the flowering period of wheat was monitored with spore traps and the effects on FHB and the mycotoxin content in the harvested grain of wheat were quantified.

Materials and Methods

Fungal strains

The *F. graminearum* strains 0410 (CBS 121292; Westerdijk Fungal Biodiversity Institute, the Netherlands), 2113 and 1145 (Fungal Collection of Agroscope, Switzerland) used in this study were isolated from diseased wheat heads in Switzerland and all identified as producers of DON belonging to the 15-acetyl-deoxynivalenol genotype following the methods described by Pasquali *et al.* (2011) for 0410 and by Quarta *et al.* (2006) for 2113 and 1145. The European antagonistic strains, including *Cladosporium cladosporioides* 761, *Clonostachys rosea* 016, *Trichoderma gamsii* CBS 120073, *T. harzianum* T-22, *Trichoderma koningii* CBS 850.68, *Trichoderma velutinum* CBS 130512 and *Trichoderma* sp. 12004, were previously described in Schöneberg *et al.* (2015). *Trichoderma atrobrunneum* ITEM908 was provided for the field experiment, because of its inhibiting effect on the perithecia development by *F. graminearum* (Altomare *et al.* 2017; Fanelli *et al.* 2018). Fresh cultures were regularly prepared on potato dextrose agar (PDA; 17·5 g l^{-1} Oxoid PDA and 7·5 g l^{-1} agar-agar) and grown for 7–14 days at 18°C in a 12/12 h NUV (black light blue tubular fluorescent lamps, wavelength 365 nm, Osram, Munich, Germany)/dark rhythm (for *F. graminearum*) or at 25°C without light (for antagonists). Between experiments, all isolates were kept as single-conidia cultures on 5 mm plugs of PDA, in 50% glycerol (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) at −70°C.

In vitro assay on perithecia development and ascospore discharge

Maize stalk pieces were prepared by collecting residues from a conventionally managed field of maize hybrid PO4044 (Pioneer Hi-Bred SL, La Rinconada, Spain, respectively) after harvest, then dried and cut into pieces of 8 cm length, containing one node each and longitudinally split in halves. At the beginning of the experiment, the maize stalk pieces were soaked with water for 48 h and subsequently autoclaved twice in fresh water for 15 min. For inoculations, macroconidia suspensions of *F. graminearum* 0410 and the antagonists were prepared by flushing fresh cultures on PDA with sterile deionized water containing 0·02% Tween20 (Riedel-de-Häen, Sigma-Aldrich GmbH), and gently scraping off the macroconidia using a Drigalski glass rod. The concentrations of the stock suspensions were determined with a haemocytometer (Thoma; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and then adjusted to 2×10^5 or 10^6 conidia per ml for the pathogen or the antagonists, respectively. Treatments with antagonists were applied 48 h before, 48 h after or simultaneously with the *F. graminearum* inoculation. Stalks were immerged in 200 ml conidial suspension in glass beakers and stirred for 5 min at room temperature. The simultaneous treatments were applied as equal mixtures of antagonist and pathogen suspensions. For the control, maize stalks were only inoculated with the pathogen. Subsequently, two maize stalk pieces from each treatment were transferred to a Petri dish (∅ 14·5 cm; Greiner Bio-One, Kremsmünster, Austria) filled with 40 g of water-saturated and autoclaved vermiculite (Isola Vermiculite AG, Switzerland). The stalks were incubated at 18°C in 12/12 h NUV/dark rhythm. After 8 weeks, all plates were placed on a grid of 1 cm^2 units and developing perithecia were counted, covering a total area of 3 cm^2, including the node and the internodes to both sides. The mean number of perithecia cm^{-2} was recorded. After counting, 2 ml of sterile water were introduced to increase the moisture. Two weeks later, the ascospore discharge was determined by washing off the ascospores discharged to the lid of the Petri dish using 1·4 ml sterile deionized water and counting as described by Schöneberg *et al.* (2015).

Formulation

Two formulation types, solid (wettable powder) and oil-based (oil adjuvant), were prepared for the experimental evaluation of *C. rosea* and *T. atrobrunneum* under field conditions.

Wettable powder of C. rosea

For the production of fungal biomass based on a solid-state fermentation, peeled barley grains (Biomill SA, Herzogenbuchsee, Switzerland) were ground with a grain mill (Cyclotec 1093 sample mill, Foss Tecator, Höganäs, Sweden), using a 1 mm screen. The moisture content of the barley flour (initially 11%) was increased to approximately 30%, by adding 400 ml water kg^{-1} of flour under constant stirring (Eurostar 60 control; 100–150 rev min^{-1}; IKA Werke GmbH & Co. KG, Staufen im Breisgau, Germany). Batches of 350 g were prepared in polypropylene zipper filter bags (32 × 57 cm; PP75/BEU6/X32-57; Sac0$_2$ NV,

Deinze, Belgium) and sealed with a welding machine (Polystar 418M-RPA, Saropack AG, Rorschach, Switzerland). All batches were autoclaved for 30 min. For each bag, the substrate was inoculated with 8 ml of a *C. rosea* conidia suspension (10^7 conidia per ml sterile water) in a laminar flow hood and incubated at 25°C in a 12/12 h NUV/dark rhythm. Over the entire incubation period, the bags were knead once a week to ensure even colonization by *C. rosea*. After 4 weeks, the fully colonized substrate was broken up manually, removed from the bags and air-dried for 3 days in aluminium trays in a laminar flow hood. To obtain the final formulation, the dried mixture of fungal biomass and barley flour (moisture content 8–10%) was blended with hectorite clay (110 mg g^{-1}; Bentone MA; Elementis GmbH, Cologne, Germany), a wetting agent (30 mg g^{-1}; Multiwet MO-85P; Croda Europe Ltd, East Yorkshire, UK) and a dispersant for aqueous solutions (60 mg g^{-1}; Atlox Metasperse 550S; Croda Europe Ltd), using a kitchen blender (HGB25E; Waring Commercial, Stanford, CT). Beforehand, the adjuvant ingredients were evaluated for possible negative effects on *in vitro* fungal growth by plating suspensions on PDA. The wettable powder formulation was prepared in both years of the field experiment and stored before use for a maximum of 2 weeks at 5°C in the dark in glass bottles with screw caps (Schott Duran®; Schott AG, Mainz, Germany).

Oil adjuvant formulation

The oil adjuvant formulation was developed under laboratory conditions to protect the conidia of *C. rosea* against harmful UV-B radiation (280–315 nm). The same formulation was also used with *T. atrobrunneum*. In brief, sunflower oil (88% unsaturated fatty acids and 11% saturated fatty acids; Denner AG, Zurich, Switzerland) was mixed with the UV-B absorber ethylhexyl methoxycinnamate (100 μl ml^{-1}) (Escalol 557; Ashland Inc., Covington, KY) and a blend of surfactants to enable emulsion of the oil in water (o/w) (200 μl ml^{-1}) (contains by volume: 48% Alkamuls 2003VO (Solvay S.A., Brussels, Belgium), 24% Atlox 4914, 24% Arlatone TV (Croda Europe Ltd) and 4% Breakthru (Omya AG, Oftringen, Switzerland)). The final formulations were obtained by mixing freshly prepared suspensions of the BCAs in sterile water with the oil and adjusting the concentration to 5% o/w emulsion (50 μl ml^{-1}). The oil formulations were prepared on the day of their application in both years of the field experiment.

Field experiment with infected maize crop residues

Field design

Over two consecutive years, a field experiment was conducted at the federal agricultural research station of Agroscope in Zurich, Switzerland (N 047°25′44″; E 008°31′04″). The previous crop in both years was potato and the field was ploughed before each experiment. The soil types were gleyic or pseudogleyic brown soils with 23 or 26% clay, 47 or 36% silt and 3% organic matter, respectively.

For the sowing of winter wheat, the seedbed was prepared by cultivator treatment followed by harrowing. The two wheat varieties, Forel and Levis (UFA Samen, Fenaco, Switzerland), were chosen from the Swiss list of recommended wheat varieties, because of their high susceptibility to FHB (Schaad *et al.*, 2019) and were sown at a density of 350 kernels per m^2 (140 kg ha^{-1}) and a row distance of 15·5 cm. The plot size was 3 × 6 m (1·5 m width for each variety) with adjacent side plots of the same dimensions, sown with either Levis or Forel. To avoid cross contamination between treatments, border plots sown with the winter triticale variety Larossa (UFA Samen) surrounded the entire plot. An illustration of the field design is presented in Fig. S1a.

The sowing of wheat was performed in November 2016 and in October 2017, respectively. During wheat cultivation, all plots were fertilized with a total of 141 kg ha^{-1} nitrogen and 20 kg ha^{-1} magnesium oxide, administered over three applications. At the end of tillering, a herbicide mixture (Artist: 24% flufenacet and 17·5% metribuzin; Chekker: 12·5% amidosulfuron and 1·3% iodosulfuron-methyl-sodium; Bayer Crop Science, Monheim am Rhein, Germany) was applied to control grass and broad-leaved weeds. The insecticides Audienz (48% Spinosad, Omya AG, Oftringen, Switzerland) or Karate Zeon (9·4% lambda-cyhalothrin; Syngenta Agro AG, Dielsdorf, Switzerland) were applied once at head emergence in 2017 or 2018 respectively to control cereal leaf beetles. All plots were harvested with a combine harvester in July and the grain was air-dried at 28°C to a moisture content of 12 ± 1%. The moisture content was measured with a grain analyser (GAC2100 Agri; Dickey-John Corporation, Auburn, IL) and the yield was determined by weighing. Weather data were collected from an adjacent weather station operated by the Swiss Federal Office of Meteorology and Climatology, MeteoSwiss, including temperature, relative humidity and precipitation.

Inoculated maize crop residues

The infected maize residues were prepared before the time of wheat sowing in both years. Stalk pieces were previously collected from conventionally managed silage maize fields of hybrid P8057 (Pioneer), cut into pieces of 30 cm length, containing one internode each and dried at 30°C with constant air flow for 3 weeks. The stalk pieces were then soaked in water for 48 h and subsequently sieved to remove the excess water. Batches of 350 stalks were placed in double-lined polypropylene bags (7002205; Ratiolab GmbH, Dreieich, Germany) and

autoclaved twice with a cooling period between runs. All treatments, except the negative control ('Control –') were inoculated with a conidial suspension (2×10^5 conidia per ml 0·02% Tween20) of three *F. graminearum* strains (0410; 1145; 2113) in equal amounts. The inoculum was produced in liquid V8-medium as described in Schöneberg *et al.* (2018b) and the suspension was applied in a volume of 700 ml per bag using a pressurized sprayer at 1·5 bar (Spray-Matic 5S, Birchmeyer Sprühtechnik AG, Stetten, Switzerland), while mixing the stalks to ensure a homogeneous distribution. The inoculated maize stalks were incubated for 5 days at 18 ± 2°C without light.

Application of the BCAs

To apply the treatments, the inoculated maize stalks were distributed in large metal trays (45 × 130 cm) before being sprayed evenly with suspensions of the antagonists until runoff (2 ml per stalk). There were four different *C. rosea* treatments: '*C. rosea*': unformulated spore suspension prepared by flushing cultures on PDA with sterile water containing 0·02% Tween20 adjusted to 10^7 conidia per ml; '*C. rosea* WP': the wettable powder formulation at 5 g l^{-1} (10^6–10^7 colony forming units (CFU) per ml); and '*C. rosea* + Oil' or '*C. rosea* WP + Oil,' respective mixtures with the oil adjuvant formulation at 5% o/w emulsion. Two different *T. atrobrunneum* treatments were applied: '*T. atrobrunneum*': unformulated spore suspension prepared by flushing cultures on PDA with sterile water containing 0·02% Tween20 adjusted to 10^7 conidia per ml; and '*T. atrobrunneum* + Oil': a mixture with the oil adjuvant formulation at 5% o/w emulsion. The control treatments were inoculated ('Control +') and sterile, non-inoculated ('Control –') maize stalks as well as the controls for the different formulations, 'WP formula': a suspension of the wettable powder co-formulants at 5g l^{-1} and 'Oil formula': an emulsion of the oil adjuvant formulation in sterile water at 5% o/w. The treated maize stalks were incubated for 16 days at 18 ± 2°C in the dark before the temperature was reduced to 10°C for another 5 days. After incubation, the residues were distributed in the designated plots at a density of 80 stalks per plot (40 stalks per variety) in early December 2016 and the end of November 2017, respectively (Fig. S1b).

Monitoring of *F. graminearum*

To determine the FHB disease pressure, *F. graminearum* was monitored on the plot level by the aid of spore traps as described by Schöneberg *et al.* (2018b) with minor modifications. Briefly, the spore traps consisted of a wooden board (30 × 10 cm) fixed to an iron rod through a drilled hole and a peg. An aluminium dish (15 × 11 × 4·5 cm) was mounted vertically onto the board. A second dish of the same type, attached horizontally at the end, served as protection against direct sunlight and rain. Per spore trap, a Petri dish (∅ 9 cm) filled with pentachloronitrobenzene (PCNB) agar was placed under the protective construction. The spore traps were set up between the subplots of the two winter wheat varieties at the height of the wheat heads and sampling was done on four occasions, covering the early (GS 61-65), full (GS 65) and late flowering periods (GS 65-69). After a sampling time of 18 h in the field, always from 16:00 until 10:00 of the next morning, the Petri dishes were incubated for 5 days at 18°C without light and the *Fusarium* colonies were counted.

The disease symptoms were visually assessed 2 weeks after the end of flowering, in every plot and separately for each variety. In each plot, 100 randomly selected wheat heads were counted for kernels or glumes showing typical bleaching symptoms and the number of symptomatic spikelets were used to determine the disease severity (% symptomatic spikelets per average number of spikelets).

The incidence of *F. graminearum* in the harvested grain was determined using a 'Seed Health Test' (SHT) as described in Vogelgsang *et al.* (2008). To determine the amount of DNA in the harvested grain, a quantitative PCR (qPCR) method (Schöneberg *et al.* 2018b) was applied to a subsample of 50 ± 2 mg flour that was prepared for the analysis of mycotoxins. Total DNA was extracted using the NucleoSpin 96 Plant II kit (Macherey-Nagel, Düren, Germany) and the concentration in the extract was determined as explained in Schöneberg *et al.* (2018a). All reactions were performed on a CFX96™ Real-Time PCR Detection System–IVD (Bio-Rad, Hercules, CA) in 96-well plates (Bio-Rad) and the standards or water control were measured in triplicates. The standard curve was established by preparing dilutions of plasmid DNA containing a fragment that is specific to *F. graminearum* (Nicholson *et al.* 1998) and mixing with wheat grain DNA free of *F. graminearum* to account for matrix effects. Each sample was measured in two technical replicates.

Mycotoxin analysis

Deoxynivalenol (DON) and zearalenone (ZEN) in the harvested grain were measured by competitive Enzyme-Linked Immunoabsorbent Assay (ELISA) using the reagents provided in the CelerDONv3 (MD100) and Celer ZEA (MZ670) detection kits (Tecna S.R.L., Trieste, Italy) according to the manufacturer's instructions. Before the analysis, the harvested grain samples were stored at 10°C in the dark. For the analysis, a 150 g subsample was prepared with a riffle divider (RT6.5, Retsch GmbH, Haan, Germany) and the grain was

Table 1 Number of perithecia per cm^2 and number of ascospores (± standard error of the mean) discharged to the lid of the Petri dish in response to biological control agents applied 48 h before (−48 h), simultaneously (0 h) or 48 h (+48 h) after the inoculation with *Fusarium graminearum* 0410

	Perithecia cm^{-2}			Ascospores × 10^4		
Biological control agent	−48 h	0 h	+48 h	−48 h	0 h	+48h
Control		63 ± 7			240 ± 25	
Cladosporium cladosporioides 761	4 ± 1***	33 ± 2*	56 ± 12	20 ± 9***	102 ± 20	86 ± 16**
Clonostachys rosea 016	0 ± 0***	0 ± 0***	0 ± 0***	0 ± 0***	0 ± 0***	0 ± 0***
Trichoderma gamsii 120073	0 ± 0***	5 ± 3***	32 ± 6**	0 ± 0***	3 ± 1***	209 ± 48
Trichoderma harzianum T22	3 ± 1***	31 ± 4***	45 ± 5	15 ± 8***	115 ± 27*	122 ± 20
Trichoderma koningii CBS 32570	1 ± 1***	10 ± 3***	36 ± 5**	2 ± 1***	37 ± 14***	106 ± 22*
Trichoderma sp. 12004	3 ± 1***	3 ± 1***	46 ± 11	3 ± 1***	17 ± 7***	99 ± 14*
Trichoderma velutinum CBS 30512	5 ± 1***	34 ± 6*	36 ± 5	7 ± 2***	102 ± 18*	106 ± 22*

Treatments that are significantly different compared with "Control" according to the Tukey test ($\alpha = 0{\cdot}05$) are indicated with symbols for *P* values <0·001 (***), <0·01 (**) and <0·05 (*).

milled (Cyclotec 1093 sample mill; Foss Tecator), using a 1-mm screen. The flour was stored at −20°C. To extract the mycotoxins, 5 g of flour were suspended in 25 ml solution of 70% methanol and 4% sodium chloride (Sigma-Aldrich, Chemie GmbH, Schnelldorf, Germany) in 100 ml Erlenmeyer flasks. Flasks were shaken for 10 min at 250 rev min^{-1} on a rotary shaker at RT. The suspension was filtered through pleated filter paper (Whatman Grade 595 ½; GE Healthcare, Chicago, IL) and three 600 μl aliquots of the filtrate were transferred to 1·2 ml micro tubes (Qiagen, Hombrechtikon, Switzerland) for the analysis of DON and ZEN. In cases where infection levels were particularly high and preliminary measurements exceeded 5 mg kg^{-1} DON, the extracts were diluted 1 : 5, 1 : 10, or 1 : 30 in the extraction solution before the measurement. The absorbance of the samples were measured with a spectrometer (Tecan Group, Männedorf, Switzerland) at 450 nm and the concentrations of DON and ZEN were calculated by the Ridasoft Win 1.84 software (R-Biopharm AG, Darmstadt, Germany) via a standard curve of known concentrations. The standard curve was included in every run. The limit of detection (LOD) was 0·04 and 0·01 mg kg^{-1} for DON and ZEN, respectively. Any detected values below the LOD were replaced by a constant value of LOD/2 for further analysis.

Data analysis

Statistical analysis was performed in R Studio ver. 1.1.463 (R Studio Team 2015), running on R version 3.3.3 (R Core Team 2015). All experiments were performed two times with four replicates in each experimental run and analysed by linear models using the R package lme4 by Bates et al. (2015)). Diagnostic residual plots, Q–Q plots and the Shapiro–Wilk test were used to check the assumptions of normality and the homogeneity of variances. In cases, where the assumptions were not met, data transformations were employed (Table S1). Plots and figures were created using the untransformed data with the ggplot2 package of R (Wickham 2016) and Inkscape, version 0.92.2 for mac OS (https://inkscape.org).

For the *in vitro* assay, the response variables 'perithecia' or 'ascospores' were predicted with the categorical predicting variable 'treatment'. The replicates nested within the experimental repetitions, were set as the random effect. Analysis of variance (ANOVA) was carried out to test the effect of 'treatment' and followed by post hoc pairwise comparisons using the Tukey test ($\alpha = 0{\cdot}05$).

For the field experiment, the data were analysed separately for each year, due to the observed differences between 2017 and 2018. The experiment was set up as a fully randomized complete block design with split plots for the factor 'variety'. In 2017, the observations for two subplots of variety Levis were excluded from the analysis due to water damage within the plot ($n = 78$). The number of *F. graminearum* colonies on PCNB was predicted with the categorical variables 'treatment' and 'sampling date', as well as their interaction and followed by ANOVA. Post hoc pairwise comparisons were performed using the Tukey test ($\alpha = 0{\cdot}05$). The response variables 'disease severity', '*F. graminearum* incidence', '*F. graminearum* genome copies' and the contents of 'DON' and 'ZEN' were predicted with the categorical variables 'treatment' and 'variety', as well as their interaction. To account for the design with split plots, a random effect for 'block' and 'variety' was introduced. To test the main effects and interactions, ANOVA was performed and followed by post hoc pairwise comparisons using the Tukey test ($\alpha = 0{\cdot}05$). To elucidate the overall relationships between collected samples over both years, a principal component analysis (PCA) was performed on the untransformed response variables.

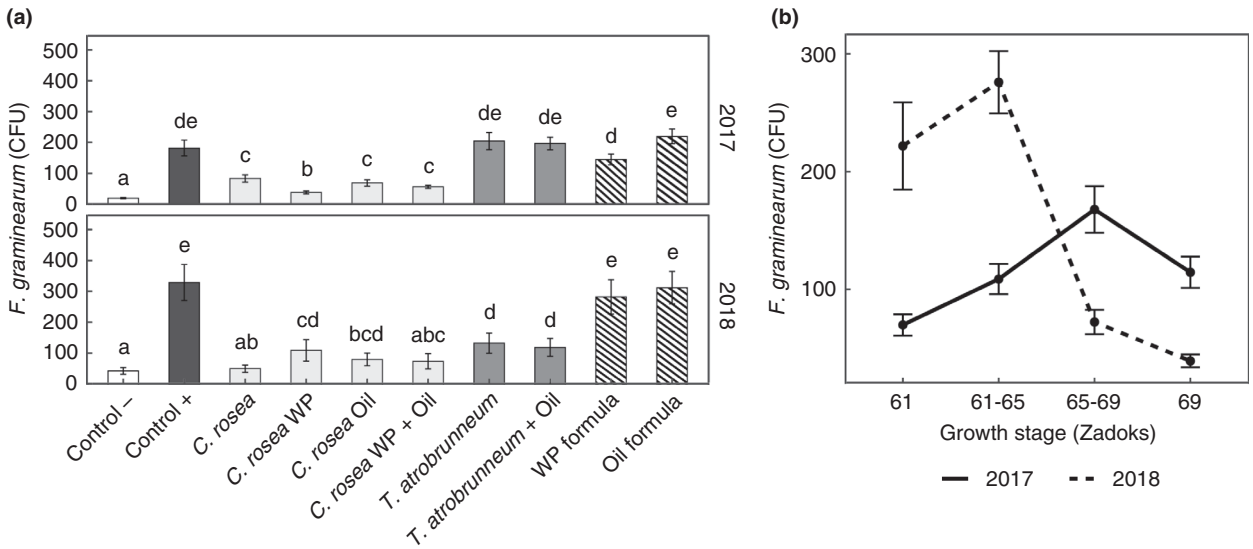

Figure 1 *Fusarium graminearum* colony forming units (CFU) on spore traps in the flowering periods of the wheat varieties Levis and Forel in 2017 (1 June to 8 June 2017) and 2018 (22 May to 28 May 2018). (a) Number of CFU in response to treatments of infected maize stalks with the biological control agents *Clonostachys rosea* (light grey bars) or *Trichoderma atrobrunneum* (grey bars). Control +/− refers to untreated inoculated/non-inoculated maize stalks (dark grey/white bars). Formula WP/Oil refers to treatments with the co-formulants of the wettable powder or the oil adjuvant formulation in sterile water respectively (black and white hatched bars). Average data from replicate plots and four sampling time points over the flowering period are presented and bars represent the standard error of the mean (n = 16). Treatments that share the same letter are not significantly different according to a Tukey test (α = 0·05). (b) Number of CFU on spore traps, averaged over treatments (n = 40) at different growth stages during the flowering of wheat in 2017 (solid line) and 2018 (dashed line)

Results

In vitro perithecia development and ascospore discharge by *F. graminearum* in response to BCAs

The quantification of perithecia development and discharge of ascospores by *F. graminearum* on sterilized maize stalk pieces showed that when BCAs were applied prior to inoculation with the pathogen, all significantly ($P < 0{\cdot}001$) reduced the mean number of perithecia and ascospores by 92–100% compared with the control treatment (on average 63 perithecia per cm^2 and 2×10^6 ascospores per Petri dish). In simultaneous applications, the perithecia formation was significantly (*P*-value range: <0·001–0·048) inhibited by *C. rosea*, *T. gamsii* and *Trichoderma* sp. (average inhibition rates between 90 and 100%), followed by *T. koningiopsis* (84%) and the other BCAs (49%). In contrast, when the application was conducted after the inoculation with *F. graminearum*, only *C. rosea* showed 100% inhibition of perithecia development ($P < 0{\cdot}001$), followed by *T. gamsii* (50%, $P = 0{\cdot}012$) and *T. koningiopsis* (44%, $P = 0{\cdot}018$). The other BCAs did not result in significant reductions, ranging between 11 and 43% (Table 1).

The Spearman's rank correlation between the number of perithecia developed per cm^2 and number of ascospores per Petri dish resulted in a correlation coefficient of $r = 0{\cdot}87$ ($P < 0{\cdot}001$). Nevertheless, variable trends for number of perithecia and ascospores were observed for *C. cladosporioides* and *T. gamsii* when applied after the inoculation with *F. graminearum*. In fact, *T. gamsii*, showing the second highest inhibition on development of perithecia (50%), resulted in the lowest reduction of ascospore discharge (13%). In contrast, *C. cladosporioides* that showed the lowest inhibition of perithecia development (11%), recorded the second highest reduction of ascospore discharge with 64% compared with the control (Table 1). The maize stalk pieces treated with *C. rosea* were fully colonized by the BCA, while the visible colonization by other BCAs was considerably lower when applied simultaneously or after the inoculation with *F. graminearum* (Fig. S2).

Biological control of *F. graminearum* in the field

Spore deposition

The monitoring of *F. graminearum* by the aid of spore traps showed a significant effect of the sampling date in 2017 and 2018 ($F_{3,\ 108} = 30$, $P < 0{\cdot}001$ and $F_{3,\ 118} = 177$, $P < 0{\cdot}001$). Furthermore, there were significant effects of the treatments (2017: $F_{9,\ 108} = 70$, $P < 0{\cdot}001$ and 2018: $F_{9,\ 118} = 51$, $P < 0{\cdot}001$). In both years, the interaction

Table 2 Fusarium head blight (FHB) disease severity (% symptomatic spikelets determined 14 days after the end of the flowering period) and the grain yield per year and wheat variety ((± standard error of the mean) $n = 4$)

	2017				2018			
	FHB disease severity (%)		Grain yield (t ha^{-1})		FHB disease severity (%)		Grain yield (t ha^{-1})	
Treatment	Forel	Levis	Forel	Levis	Forel	Levis	Forel	Levis
Control +	2·5 ± 0·4	5·2 ± 1·4	8·4 ± 0·5	8·4 ± 0·4	18·9 ± 2·3	46·6 ± 7·7	7·0 ± 0·2	5·0 ± 0·2
Control −	0·2 ± 0·1**	0·2 ± 0·1***	9·5 ± 0·4	8·7 ± 0·4	0·5 ± 0·2***	2·2 ± 0·7***	9·2 ± 0·4***	9·3 ± 0·5***
Clonostachys rosea	1·4 ± 0·6	1·7 ± 0·5	8·5 ± 0·3	8·3 ± 0·5	1·1 ± 0·4***	4·7 ± 0·7***	9·8 ± 0·3***	9·6 ± 0·3***
Clonostachys rosea WP	0·3 ± 0·1**	0·8 ± 0·3***	8·8 ± 0·2	8·1 ± 0·2	2·5 ± 1·2***	11·5 ± 1·2***	9·2 ± 0·1***	8·8 ± 0·2***
Clonostachys rosea + Oil	0·9 ± 0·4	1·2 ± 0·6**	8·8 ± 0·2	8·3 ± 0·2	2·1 ± 0·7***	5·6 ± 1·3***	9·8 ± 0·2***	9·6 ± 0·1***
Clonostachys rosea WP + Oil	0·8 ± 0·3	1·7 ± 0·8	8·8 ± 0·5	8·7 ± 0·5	1·2 ± 0·3***	7·7 ± 0·7***	9·6 ± 0·3***	9·2 ± 0·3***
Trichoderma atrobrunneum	2·4 ± 0·5	7·6 ± 2·0	8·2 ± 0·2	7·8 ± 0·2	4·0 ± 1·1***	12·8 ± 3·9***	9·0 ± 0·2***	8·0 ± 0·3***
Trichoderma atrobrunneum + Oil	2·9 ± 0·1	7·0 ± 2·1	8·7 ± 0·3	7·7 ± 0·2	2·7 ± 0·6***	10·4 ± 2·0***	9·2 ± 0·4***	8·5 ± 0·3***
WP formula	2·9 ± 0·6	5·2 ± 1·4	8·0 ± 0·6	8·1 ± 0·3	16·2 ± 2·0	50·2 ± 3·6	7·2 ± 0·3	4·9 ± 0·2
Oil formula	3·0 ± 0·6	6·7 ± 1·4	8·2 ± 0·2	7·8 ± 0·9	14·6 ± 0·9	41·0 ± 4·2	7·5 ± 0·1	5·7 ± 0·3

Treatments that are significantly different compared with "Control +" (untreated inoculated maize stalks) according to the Tukey test ($\alpha = 0{\cdot}05$) are indicated with symbols for P values <0·001 (***), <0·01 (**) and <0·05 (*).

between date and treatment was not significant. For the flowering periods of the first (01.6.–08.06.2017) and the second year (22.05.–28.05.2018), all treatments with *C. rosea* significantly reduced ($P < 0{\cdot}001$) the mean number of *F. graminearum* CFU on PCNB by up to 79% and up to 85% compared with the positive control, respectively. Comparison between treatments showed that application of *C. rosea* WP resulted in a significantly ($P < 0{\cdot}05$) lower number of CFU in 2017 compared with the unformulated spore suspension or the mixtures with oil, but not in 2018, where no difference was found compared with other *C. rosea* treatments. The treatments with *T. atrobrunneum* either as unformulated spore suspension or in emulsion with oil adjuvants showed no significant effects on CFU in 2017. However, in 2018, the treatments with *T. atrobrunneum* reduced the CFU by up to 64% compared with the positive control. The added oil adjuvant formulation resulted in no significant improvements for both BCAs. In addition, the treatments with the coformulants alone showed no significant effect compared with the positive control (Fig. 1a). In 2018, the total amount of CFU retrieved by spore traps over the flowering period was 39% higher compared with 2017. Overall, the number of CFU increased towards the end of flowering in 2017 whereas in 2018, a strong peak occurred towards the full flowering stage, followed by decreasing spore deposition (Fig. 1b).

Disease symptoms and grain yield

The analysis of disease severity in 2017 and 2018, revealed a significant effect of the variety ($F_{1,8} = 13$, $P < 0{\cdot}01$ and $F_{1,8} = 95$, $P < 0{\cdot}001$) and the treatment ($F_{9,70} = 27$, $P < 0{\cdot}001$ and $F_{9,72} = 75$, $P < 0{\cdot}001$) with no significant interaction. In 2017, the mean disease severity ranged from 0 to 8% infected spikelets, while in 2018, it was on average five times higher, ranging from 1 to 50%. In both years, the variety Levis showed significantly ($P < 0{\cdot}01$) higher levels of disease severity than the variety Forel. Corresponding with the reduction of deposited spores (CFU), the *C. rosea* WP treatment significantly reduced the disease severity ($P < 0{\cdot}01$) by 85% in 2017 compared with the positive control. The treatments combining the spore suspension of *C. rosea* or the WP with the oil adjuvant significantly ($P < 0{\cdot}001$) reduced the disease severity by 71 or 66%, respectively. The effect of an unformulated spore suspension on disease severity was lower with 58% reduction ($P > 0{\cdot}05$). Application of *T. atrobrunneum* led to a mean increase in the disease severity of 36%, although the effect was not significant. In 2018, all treatments with BCAs significantly ($P < 0{\cdot}001$) reduced the disease severity between 74 and 91% compared with the positive control. The treatments with *C. rosea* showed the highest efficacies with up to 91% reduction of disease severity, while the emulsion of *T. atrobrunneum* with the

oil adjuvant reduced disease severity by 80%. In both years, the co-formulants alone showed no effects compared with the positive control (Table 2).

The strong impact of *F. graminearum* resulted in yield reductions of 14 and 7% in 2017 or 21 and 44% in 2018 for varieties Levis and Forel, respectively, when comparing the positive with the negative controls. The BCAs showed no significant effects on the grain yield in 2017, but significantly increased yield under the high disease pressure in 2018 by up to 40% (*C. rosea* WP) in variety Forel and by up to 92% in variety Levis (*C. rosea*) compared with the positive control (Table 2).

FHB species composition and fungal biomass in harvested grain

Fusarium graminearum was the dominant fungal species in both years, with a mean relative abundancy of 92% in 2017 and 96% in 2018 among the FHB species complex. The mean percentages of grain infected with *F. graminearum* ranged from 4 to 44% in 2017 and from 20 to 89% in 2018 (Table 3). The higher susceptibility of variety Levis compared with Forel was reflected in a significantly higher level of grain infection in both years with +57% ($P = 0{\cdot}001$) and +32% ($P = 0{\cdot}008$), respectively. Besides *F. graminearum*, the second and third most abundant FHB species were *F. poae* with 2–6% mean incidences, followed by *Microdochium* spp. with 1–3%. Neither the treatment nor the variety showed an effect on the incidence of *Microdochium* spp., but in both years, the incidence of *F. poae* was significantly (2017: $F_{1,8} = 7$, $P < 0{\cdot}01$ and 2018: $F_{1,8} = 16$, $P < 0{\cdot}05$) higher in grains from the variety Levis compared with Forel. Compared with the positive control, the *C. rosea* treatments in 2017 resulted in up to 65% reduction of *F. graminearum* incidence in grains of the variety Levis and up to 68% reduction in grains from the variety Forel. No significant differences were detected between the different formulations of the BCA. In 2018, the *C. rosea* treatment significantly reduced ($P < 0{\cdot}001$) the incidence of *F. graminearum* in grains from the variety Forel and Levis by up to 69 and 53%, respectively. The application of *T. atrobrunneum*, in combination with the oil adjuvant formulation, significantly reduced the *F. graminearum* incidence in Forel and Levis by up to 52 and 36%, respectively. The co-formulants showed no significant effect compared with the positive control. As for the incidence, the analysis by qPCR revealed a significant effect of the variety ($F_{1,78} = 23$, $P < 0{\cdot}001$ and $F_{1,80} = 73$, $P < 0{\cdot}001$) and the treatment ($F_{9,78} = 35$, $P < 0{\cdot}001$ and $F_{9,80} = 89$, $P < 0{\cdot}001$) on the detected number of *F. graminearum* genome copies in the harvested grain for 2017 and 2018 (Table 3).

Deoxynivalenol and zearalenone content in harvested grain

As for the disease symptoms and the fungal incidence in grains, the DON content revealed elevated

Table 3 Number of *Fusarium graminearum* genome copies and the infection incidence (± standard error of the mean) based on a seed health test (SHT) in the harvested grains per year and wheat variety ($n = 4$)

	2017				2018			
	Fusarium graminearum DNA (copies ng^{-1} extracted DNA) × 100		*Fusarium graminearum* Incidence in SHT (%)		*Fusarium graminearum* DNA (copies ng^{-1} extracted DNA) × 100		*Fusarium graminearum* Incidence in SHT (%)	
Treatment	Forel	Levis	Forel	Levis	Forel	Levis	Forel	Levis
Control +	5·5 ± 0·2	6·7 ± 1·1	24 ± 2	39 ± 4	23·3 ± 5·0	44·5 ± 7·8	79 ± 5	85 ± 6
Control −	1·0 ± 0·3**	0·5 ± 0·1***	5 ± 1**	4 ± 1***	1·1 ± 0·4***	2·6 ± 0·6***	20 ± 8***	29 ± 9***
Clonostachys rosea	2·8 ± 0·9	2·8 ± 0·6	15 ± 3	18 ± 2**	1·6 ± 0·4***	3·8 ± 0·8***	24 ± 4***	43 ± 3***
Clonostachys rosea WP	1·9 ± 0·3	2·0 ± 0·3**	9 ± 1*	14 ± 1***	3·8 ± 1·2***	9·8 ± 1·0***	31 ± 5***	53 ± 4***
Clonostachys rosea + Oil	2·3 ± 0·4	2·6 ± 0·8*	13 ± 4	18 ± 4***	2·5 ± 0·6***	3·9 ± 0·9***	30 ± 8***	39 ± 6***
Clonostachys rosea WP + Oil	2·5 ± 0·3	3·3 ± 0·6	8 ± 2*	19 ± 2**	2·7 ± 0·3***	5·1 ± 0·5***	27 ± 2***	46 ± 2***
Trichoderma atrobrunneum	6·1 ± 0·1	9·1 ± 0·8	26 ± 2	36 ± 5	5·1 ± 1·3***	8·1 ± 2·1***	43 ± 7**	58 ± 8*
Trichoderma atrobrunneum + Oil	6·3 ± 1·1	10·3 ± 0·8	24 ± 3	44 ± 4	3·7 ± 0·4***	6·8 ± 0·4***	38 ± 2***	54 ± 6**
WP formula	5·7 ± 0·9	8·3 ± 1·4	25 ± 5	37 ± 5	18·9 ± 4·5	40·1 ± 3·1	72 ± 7	89 ± 1
Oil formula	6·6 ± 1·4	10·0 ± 1·6	20 ± 2	44 ± 5	23·9 ± 6·0	34·0 ± 5·0	72 ± 6	80 ± 4

Treatments that are ignificantly different compared with "Control +" (untreated inoculated maize stalks) according to the Tukey test ($\alpha = 0{\cdot}05$) are indicated with symbols for *P* values <0·001 (***), <0·01 (**) and <0·05 (*).

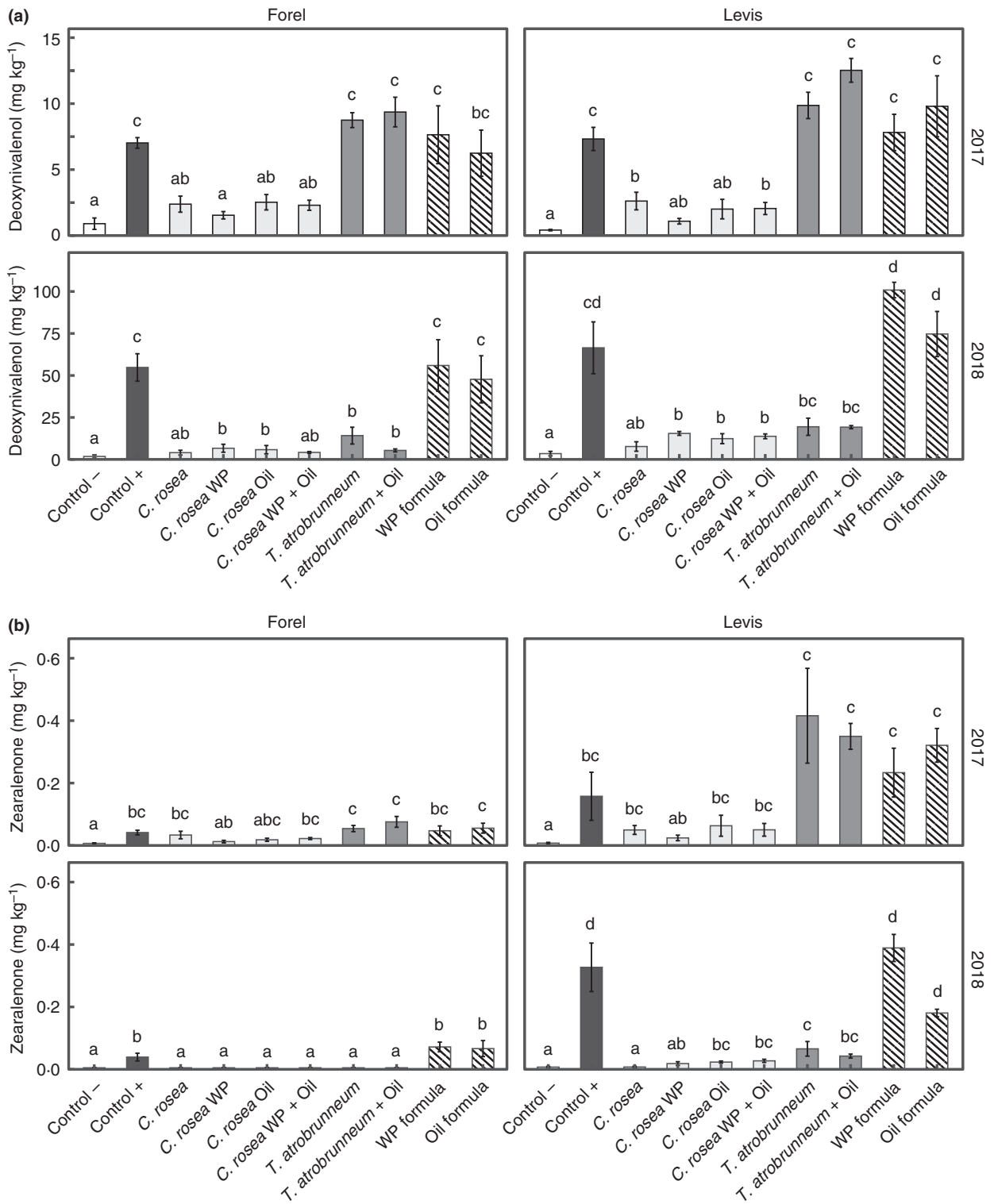

Figure 2 (a) Deoxynivalenol (mg kg^{-1}) and (b) zearalenone (mg kg^{-1}) contents in harvested wheat grain of winter wheat varieties Forel and Levis in 2017 and 2018 in response to treatment of infected maize crop residues with formulations of the biological control agents, *Clonostachys rosea* (light grey bars) or *Trichoderma atrobrunneum* (grey bars). Control +/– refers to untreated inoculated/non-inoculated maize stalks (dark grey/white bars). Formula WP/Oil refers to treatments with co-formulants in sterile water (black and white hatched bars). Average data from four replicates are presented and bars represent the standard error of the mean (n = 4). For each variety and year, treatments that share the same letter are not significantly different from each other according to the Tukey test (α = 0·05).

contaminations under the higher disease pressure of 2018. In 2018, the variety effect was significant, with variety Levis showing on average higher ($F_{1,80} = 52$, $P < 0{\cdot}001$) DON accumulation compared with Forel; however, the contents of the positive control treatments were not substantially different between varieties. In both years, there was no interaction between the variety and the treatment. The treatments showed significant influence on the mean DON contamination, ranging between 0·4 mg kg^{-1} (Control −) and 12·5 mg kg^{-1} (*T. atrobrunneum* + Oil) in the first and between 1·8 mg kg^{-1} (Control −) and 100·9 mg kg^{-1} (WP formula) in the second year. In 2017, *C. rosea* treatments led to a reduction of the mean DON content between 64 and 85% in grains from the variety Levis and between 66 and 78% in grains from the variety Forel. In 2018, the mean DON content reductions ranged between 77 and 88% and between 88 and 93%, respectively. The treatments with *T. atrobrunneum* showed no significant difference to the positive control in the first year, but significantly reduced the DON content in the second year between 74 and 90% in the variety Forel and by 71% in the variety Levis. No differences were found between the formulations of the BCAs. The effects of the co-formulants of the WP or the oil adjuvant formulas without a BCA compared with the positive control were not significant (Fig. 2a).

The average accumulation of ZEN was higher in 2017 compared with 2018. In both years, grains from the variety Levis showed significantly higher (2017: $F_{1,78} = 20$, $P < 0{\cdot}001$; 2018: $F_{1,80} = 249$, $P < 0{\cdot}001$) ZEN contents compared with grains from Forel. The mean ZEN content in the harvested grain ranged between 0·01 mg kg^{-1} (Control −) and 0·42 mg kg^{-1} (*T. atrobrunneum*) in the first and between <0·01 mg kg^{-1} (Control −) and 0·39 mg kg^{-1} (WP formula) in the second year. In 2017, only the treatment with *C. rosea* WP significantly reduced the ZEN content compared with the positive control by 84% in grains from the variety Levis and by 78% in grains from the variety Forel, while in 2018, all BCA treatments resulted in significantly reduced ZEN contamination. No differences were found between the formulations (Fig. 2b).

Correlations between response variables from the field experiment

The result from the PCA on the response variables was presented as a biplot, showing the two most descriptive dimensions of the variables (Fig. 3). The first dimension, with 60% contribution to the overall variance, shows the relationship between spore deposition, disease symptoms in the field, the DON and ZEN contents, *F. graminearum* genome copies and incidence of *F. graminearum* in the harvested grains that were strongly negatively correlated with the grain yield. The incidence of *Microdochium* spp. was only slightly associated with grain yield and negatively correlated with the incidence of *F. poae* along the second dimension, contributing 12% to the overall variance.

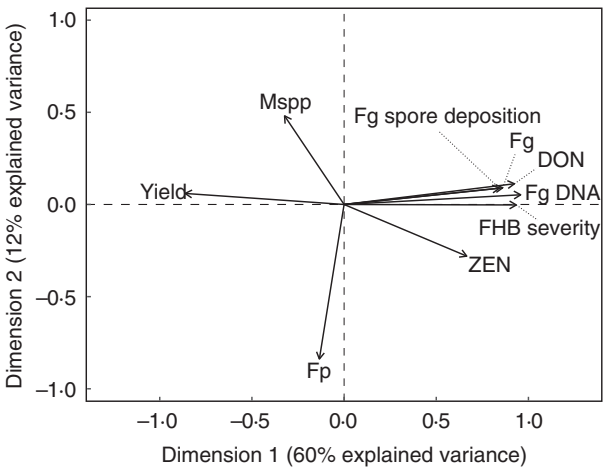

Figure 3 Biplot of the principal component analysis (PCA) on *Fusarium graminearum* spore deposition (Fg spore deposition), disease symptoms (FHB severity), number of *F. graminearum* genome copies (Fg DNA), deoxynivalenol contents (DON) and zearalenone contents (ZEN), fungal species incidence in the harvested grain for *F. graminearum* (Fg), *Fusarium poae* (Fp) and *Microdochium* spp. (Mspp) as well as the grain yield (Yield) in samples from the field experiment. $n = 155$.

Discussion

The inoculum of *F. graminearum* from infected crop residues that remain on the field, especially of maize, is the primary source for FHB and causes severe contamination of the subsequent crop with mycotoxins (Blandino *et al.* 2010). Therefore, the biological control of *F. graminearum* on crop residues represents a highly desirable alternative to the use of chemical fungicides that must be applied directly on the crop. With this study, we present a 'lab to field' approach that provides a solid proof of concept with the BCA, *C. rosea*, able to suppress the primary inoculum in a simulated maize-wheat crop rotation over 2 years, which led to reduced contaminations of wheat grain with DON and ZEN.

A wide range of fungal competitors is known that outcompete *F. graminearum* on wheat and maize substrates under different conditions, which includes primarily members of the genera *Trichoderma* and *Clonostachys*, but also *Cladosporium* and *Microsphaeropsis* (Bujold *et al.* 2001; Luongo *et al.* 2005; Inch and Gilbert 2007). In this study, the strong effect on the already *F. graminearum*-infected maize stalks separates *C. rosea* from other tested BCAs including *C. cladosporioides* and several *Trichoderma*

species, whom showed high competitiveness against *F. graminearum*, when applied before and simultaneously with the pathogen, but significantly less after the infection in the laboratory. This finding is in line with the study by Schöneberg *et al.* (2015), conducted under comparable conditions who observed a significant inhibition of perithecia development by 90, 74 and 73% and consequently the full inhibition of ascospore release when *C. rosea* was applied on wheat haulm pieces, before, simultaneously with or after the infection with *F. graminearum*, respectively. Interestingly, the even higher reduction of perithecia development observed on maize stalks compared with wheat haulms, could be the result of differences between the substrates. As seen by Khonga and Sutton (1988), the perithecia production on crop residues is strongly influenced by the decomposition process, whereas, the decomposition of maize residues, is slower than that of wheat haulms, resulting in delayed perithecia development. This and the fact that the mycoparasitic agent *C. rosea* requires direct contact with *F. graminearum* to be most effective (Schöneberg *et al.* 2015), may explain the increased suppressive effect with the extended time before *F. graminearum* begins its sexual reproduction on maize stalks. Understanding the compatibility of *C. rosea* with different host crop residues is valuable for the implementation of a practical BCA strategy.

Compared with the substantial number of known antagonists against *F. graminearum*, little information is available about the effect of BCA treatments on the airborne inoculum under field conditions. The estimation of airborne spores within and around the field has been widely used to study the epidemiology of *F. graminearum* (Schaafsma *et al.* 2005; Hellin *et al.* 2018; Schöneberg *et al.* 2018b). We utilized easy-to-built and efficient spore traps during the flowering period of winter wheat, which enabled the comparison of spore deposition between treatments and provided an insight into the dynamics of the disease pressure. The high FHB severity of 2018 matched higher total amounts of deposited spores and a strong peak at the full flowering stage of the wheat, when the crop is highly susceptible (Osborne and Stein 2007). The gathering of information on the relative abundancy of airborne inoculum is further advantageous, thanks to its correlation with all other disease-related parameters in the field as well as in the harvested grain. Here, the consistent effect of *C. rosea* selected *in vitro*, reducing not only the airborne inoculum but also the accumulation of mycotoxins, shows the excellent potential of the BCA to survive and suppress the pathogen on crop residues when exposed to field conditions over winter and spring. Similarly, Luongo *et al.* (2005) found between 60 and 90% reduction of *F. graminearum* conidia production on maize stubbles treated with *C. rosea*.

Unlike *C. rosea*, the treatments with *T. atrobrunneum* (formerly *T. harzianum*) showed inconsistency between the two years of the field experiment. The significant reduction of the spore deposition by up to 64% and a reduced disease development only occurred under the high disease pressure of the second year, that is, 2018. Previously, the application of *T. harzianum* on wheat straw significantly reduced the incidence of *F. graminearum* and inhibited the production of perithecia by up to 92% after 60 days of field exposure during spring and summer (Fernandez 1992; Inch and Gilbert 2007). The observed difference between the two years of our field experiment is possibly due to climatic conditions that benefited *T. atrobrunneum* over *F. graminearum*, with on average higher daily temperatures (+1°C) and increased precipitation (+73 mm) throughout the winter and spring periods in 2018 compared with 2017 (Table S2). *In vitro*, the growth and most of the enzymatic activities by *T. harzianum* are correlated with increased temperature and increased water potential (Kredics *et al.* 2000). Detrimental effects of low water potential and temperature on *T. harzianum* in the interaction between decomposing fungi on cereal residues were observed by Magan and Lynch (1986). Singh *et al.* (2009) found significant variation in antagonistic activity of *T. harzianum* against *Fusarium pseudograminearum* on wheat straw under changing conditions, being the most effective under wet conditions and medium to high temperatures.

The different formulations of BCAs tested in our study showed no substantial differences in the efficacies against *F. graminearum,* compared with the application of unformulated, freshly harvested spores. In case of *C. rosea*, this finding is supported by the results of Jensen *et al.* (2000), where the formulation of the BCA as a mixture of fungal biomass, wheat bran and sphagnum peat in water did not perform significantly different compared with freshly produced spores against seed infection by *F. culmorum*. The authors also compared the efficacies between the application of spores formulated with the adjuvant sticker Pelgel and the blank formulation without *C. rosea* on seeds and found no significant effect of the co-formulant on the pathogen. This observation is in line with our treatments of infected crop residues with blank formulation of the barley grain-based wettable powder or the emulsion with oil and UV protectant, which did not lead to significant effects on *F. graminearum* compared with the positive control. However, it remains to be investigated whether the addition of UV protectants solubilized in oil could contribute to the establishment of the BCAs following a direct application on residues in the field, where UV-B radiation can reach levels that are detrimental to the establishment of soil-borne fungi (Kaiser *et al.* 2019). In general, information on formulation of BCAs is scarce, due to commercial interests behind their development.

Thus, the reported efficacy of *C. rosea* in form of a wettable powder, produced in solid-state fermentation on barley grains, suggests an easy to handle alternative to freshly produced spores from the laboratory.

Clearly, the most important FHB related parameter for growers and buyers of wheat is the accumulation of mycotoxins in response to agronomic practices and the prevailing environmental conditions. By simulating a system with high disease pressure, we provided a reproducible platform to elucidate the potential of BCAs to prevent the contamination of wheat grain with DON and ZEN. Although both winter wheat varieties were highly susceptible to *F. graminearum* infection, Forel showed significantly less disease symptoms and significantly higher resistance to the accumulation of ZEN. As a result, the presumably lower yielding Forel outperformed Levis in terms of grain yield under high disease pressure, because of the reduced impact in the less susceptible variety. The substantially higher contamination of grain with DON in 2018 was correlated with increased relative humidity (+6%) and precipitation (+28 mm) during flowering until the early dough stage. The opposite was the case for the accumulation of ZEN, which was lower in 2018 compared with 2017. These results can be explained by the relatively wet conditions after the infection until the harvest. In 2017, the precipitation was twice as high in the late stages of wheat production compared with 2018, which was shown to increase ZEN in wheat grain (Edwards 2011; Drakopoulos *et al.* 2020) (Table S2).

In conclusion, our findings present a strong point for further development of a control strategy against *F. graminearum* using mycoparasitic fungi, especially *C. rosea* as a biological crop residue treatment. The efficacy on maize stalks under *in vitro* conditions and the sustained activity of *C. rosea*, once established, encourages the development of a suitable application strategy adapted to grower's needs. It remains to clarify the best timing and conditions for the application under field conditions, considering the effects of temperature and moisture in autumn compared with spring. Furthermore, it is crucial to study whether infected crop residues must be treated before, early or late during the wheat production to achieve disease control under natural disease pressure. A highly promising direction in future studies could be the combination of the commonly practiced mulching of maize crop residues before sowing with a spray application of an indigenous strain of *C. rosea* to promote establishment of the fungus not only on, but also within infected crop residues to suppress *F. graminearum*.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Illustration of one replicate block for the field experiment with the winter wheat varieties Forel and Levis.

Figure S2. Maize stalk pieces in response to treatments with biological control agents (BCAs), namely *Cladosporium cladosporioides* strain 761, *Clonostachys rosea* strain 016, *Trichoderma gamsii* strain CBS 120073, *T. harzianum* strain T-22, *T. koningii* strain CBS 850.68, *Trichoderma* sp. strain 12004 and *T. velutinum* strain CBS 130512, applied 48 h before (−48 h), simultaneously (0 h), or 48 h (+48 h) after the inoculation with *Fusarium graminearum* strain 0410.

Table S1. Transformations for the response variables (Y) from the *in vitro* assay on perithecia development and ascospore discharge and the field experiment with infected maize crop residues to meet the assumptions of linearity.

Table S2. Average temperature (°C), average relative humidity (%) and sum of precipitation (mm) during different growing stages of wheat in 2017 and 2018: winter and spring periods (growth stages 00–59), flowering period (growth stages 61–69), kernel water ripe until early dough (growth stages 71–83) and soft dough until ripening (growth stages 85–92).